RESEARCH LETTER

Occurrence of the polyamines caldopentamine and homocaldopentamine in axenic cultures of the red tide flagellates *Chattonella antiqua* and *Heterosigma akashiwo* (*Raphidophyceae*)

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Abstract

The polyamines caldopentamine and homocaldopentamine were detected in axenic strains of *Chattonella antiqua* and *Heterosigma akashiwo* (*Raphidophyceae*), respectively, as well as spermidine, the most abundant polyamine in both phytoplankton species. Trace amounts of putrescine, diaminopropane and norspermine were also detected in both species. Spermine was detected only from *C. antiqua*. These long linear polyamines are characteristic components of thermophilic bacteria. The detection from two species of *Raphidophyceae* indicates that the occurrence of long linear polyamines is not restricted to thermophilic microorganisms.

Introduction

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous components of living organisms and these three compounds are often referred as common polyamines. These polyamines are one of the important growth-regulating elements involved in protein synthesis through binding to DNA and RNA (Tabot & Tabor, 1984; Igarashi & Kashiwagi, 1999). Polyamines are also detected from microalgae and some implications with their growth are suggested (Aleksijevic *et al*., 1979; Hamana & Matsuzaki, 1985; Hamana *et al*., 2004; Nishibori & Nishijima, 2004; Nishibori *et al*., 2006).

Long linear polyamines, such as caldopentamine and homocaldopentamine, together with branched polyamines, were originally discovered in extreme thermophilic bacteria (Oshima, 1978; Oshima & Kawahata, 1983). The cellular concentrations of these uncommon polyamines in *Thermus thermophilus* correlate with the growth temperature (Oshima, 1989), and are essential for protein synthesis at the physiological temperatures of the thermophiles' natural environment. The reported function of these uncommon polyamines, which are essential for the stabilization of nucleic acids, suggests that they play an important role in the maintenance of cellular function at high temperatures in thermophilic bacteria (Ohno-Iwashita *et al*., 1975).

Harmful algal blooms caused by active and exceptional growth of microalgae occur around the coast of the world (Edvardsen & Imai, 2006). Harmful algal blooms have caused damage such as mass mortalities of fishes, marine mammals, and shellfishes, and also illness and death in humans consuming contaminated specimens. *Chattonella antiqua* and *Heterosigma akashiwo*, belonging to *Raphidophyceae*, are typical organisms leading to fish kill blooms (Honjo, 1994; Edvardsen & Imai, 2006). Here, we report a new finding of the occurrence of caldopentamine and

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homocaldopentamine in axenic clonal cultures of the red-tide-causing microalgae *C. antiqua* and *H. akashiwo*.

Materials and methods

Sample preparation

The polyamines diaminopropane, putrescine, cadaverine, spermidine, spermine, norspermidine, and norspermine were used as standard polyamines. One milliliter of 40 μM standard mixture in 5% trichloroacetic acid (TCA) was benzoylated after being alkalized with NaOH as described by Taibi & Schiavo (1993).

Axenic clonal strains of *C. antiqua* (NIES-1) and *H. akashiwo* (NIES-6) were cultured in 1 L of ESM medium (Okaichi *et al.*, 1982) under 70 μmol photons m^{-2} s^{-1} at a temperature of 20 °C until late exponential phase. The cells collected by centrifugation at 200 ***g*** for 10 min were added to a portion of 5% TCA and sonicated to disrupt the cells. The supernatant obtained by centrifugation was applied to a Dowex 50W column as described by Hamana (1993) to remove amino acids, and polyamines were eluted with 6 M HCl. The polyamine fraction was dried, resuspended into 1 mL of 5% TCA, and benzoylated as described above (Taibi & Schiavo, 1993).

Polyamine identification

The benzoylated samples were analyzed using an Alliance HPLC system (Waters Co., MA) coupled to an LCQ Freet mass spectrometer (Thermo Fisher Scientific, MA) fitted with an ESI interface. Separations were performed on a column of Capselpack C8 DD (3 mm i.d. × 150 mm, Siseido, Tokyo, Japan), and 62% methanol was used as an eluent at a flow rate of 0.2 mL min^{-1}. The voltage on the ESI interface was maintained at *c.* 5.0 kV. The temperature of the heated capillary was 275 °C. The polyamine fraction of *H. akashiwo* was also subjected to GC-MS analysis after derivatization with heptafluorobutyric anhydride and analyzed by a GC-9A gas chromatograph (Shimazu, Kyoto, Japan) and JMS-700 mass spectrometer (Jeol, Tokyo, Japan) operated in the electron impact mode at an ionization energy of 70 eV as described by Niitsu & Samejima (1993).

The caldopentamine and homocaldopentamine used were synthesized in our laboratory (Niitsu & Samejima, 1986).

Cellular contents of the long linear polyamines

Axenic clonal strains of *C. antiqua* and *H. akashiwo* were cultured as described above. A portion of the culture was withdrawn every 2–3 days and the cells were collected by centrifugation. To the resulting cell pellet, 1 mL of 5% TCA was added, sonicated and stored under − 30 °C until polyamine analysis. Samples were thawed at room temperature, centrifuged and the supernatant containing free polyamines was analyzed using HPLC with a cation exchange column 2619F (2.6 mm i.d. × 50 mm, Hitachi, Tokyo) as described elsewhere (Nishibori *et al.*, 2001).

Results

Typical HPLC chromatograms of *C. antiqua* and *H. akashiwo* are shown in Fig. 1. Benzoylated spermidine, which showed the sodium-addicted molecular ion of *m*/*z* 480, was detected both in *C. antiqua* and *H. akashiwo* as one of the major polyamines. Mass chromatograms and their spectra showed that diaminopropane (*m*/*z* 305), putrescine (*m*/*z* 319) and norspermine (*m*/*z* 627) were the minor polyamine components in both species, although their existence was not shown clearly in the chromatograms from HPLC. Spermine (*m*/*z* 641) was detected in *C. antiqua*, but not from *H. akashiwo* by mass chromatogram.

Unknown peaks were eluted after spermine from *C. antiqua* and *H. akashiwo*, respectively. The unknown peak detected in *C. antiqua* showed a protonated molecular ion peak at *m*/*z* 766, which is identical to the authentic caldopentamine (Fig. 2). The LC-MS/MS analysis revealed the major fragment ions, identical to the authentic compound, at *m*/*z* 644, 484, and 323 (Fig. 3). From the analysis of these fragment ions, the unknown peak from *C. antiqua* was identified as caldopentamine (Fig. 3). Another unknown peak eluted from *H. akashiwo* showed a protonated molecular ion at *m*/*z* 780, suggesting homocaldopentamine or thermopentamine (Fig. 2). The GC-MS analysis, which can separate heptafluorobutyryl derivatives of these two

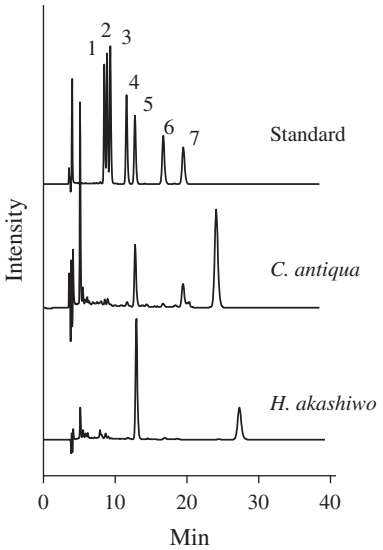

Fig. 1. HPLC profiles of standard polyamines, polyamines in *Chattonella antiqua* and *Heterosigma akashiwo*. 1, putrescine; 2, diaminopropane; 3, cadaverine; 4, norspermidine; 5, spermidine; 6, norspermine; and 7, spermine.

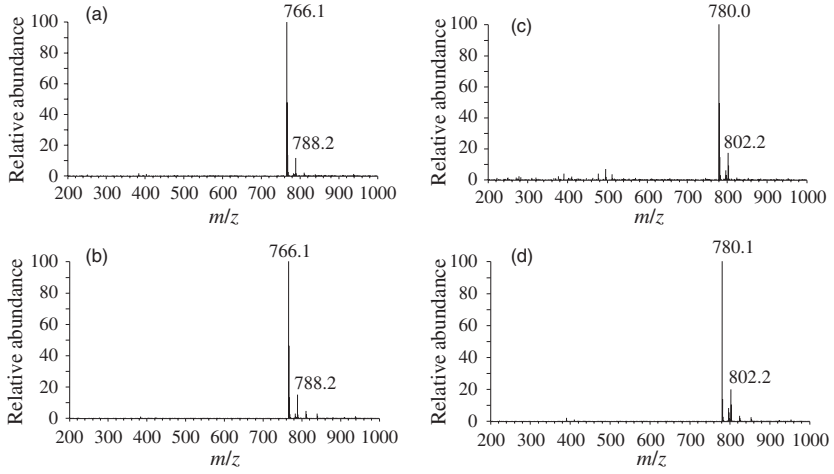

Fig. 2. Mass spectra of unknown polyamines occurred in (a) *Chattonella antiqua*, (b) *Heterosigma akashiwo* and (c) synthetic caldopentamine and (d) homocaldopentamine.

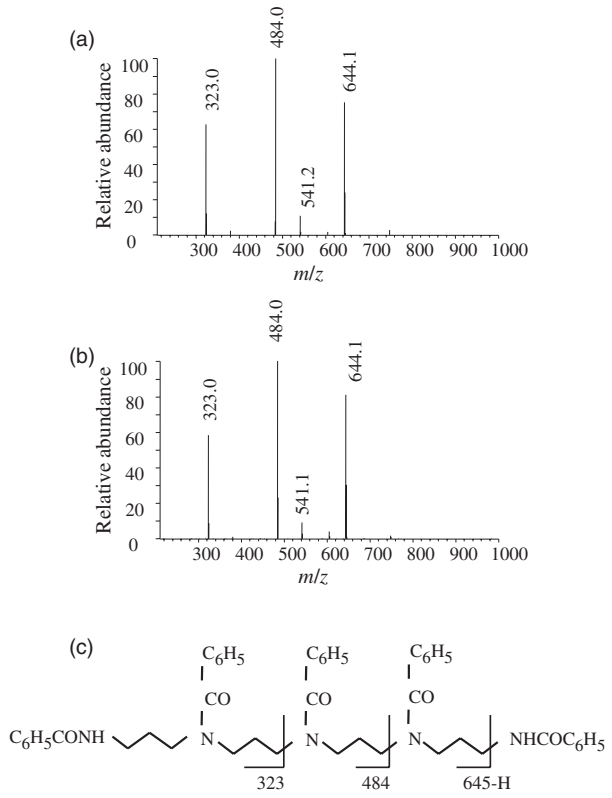

Fig. 3. MS-MS spectrum of unknown polyamines in (a) *Chattonella antiqua* and (b) synthetic caldopentamine. (c) MS/MS fragmentation was also proposed.

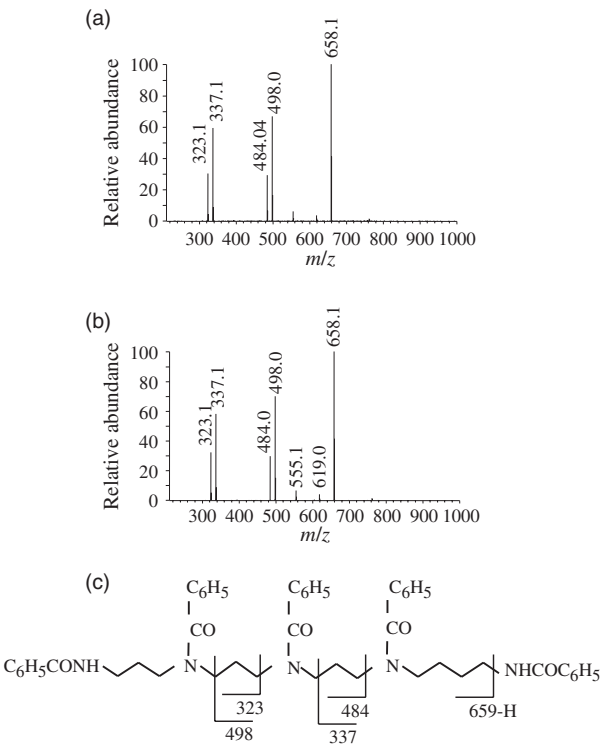

Fig. 4. MS-MS spectrum of unknown polyamines in (a) *Heterosigma akashiwo* and (b) synthetic homocaldopentamine. (c) MS/MS fragmentation was also proposed.

polyamines on the chromatogram, yielded the $[M\text{-}C_3F_7]^+$ ion peak at *m*/*z* 1070 and other fragment ions. This cleavage pattern together with *m*/*z* 323, which indicates an aminobutyl end, revealed that the unknown peak from *H. akashiwo* was homocaldopentamine. The LC-MS/MS analysis of the unknown benzoylated polyamine of *H. akashiwo*, which yielded the identical fragment ions to authentic homocaldopentamine at *m*/*z* 659, 498, 484, 409, and 337, also showed that the unknown polyamine is homocaldopentamine (Fig. 4).

The cellular contents of caldopentamine in *C. antiqua* and homocaldopentamine in *H. akashiwo* are shown in Fig. 5, together with the cell densities. The content of free caldopentamine in *C. antiqua* showed a marked increase followed by a fall during the exponential growth phase and was almost constant or not detected during the stationary phase. In *H. akashiwo*, the cellular content of

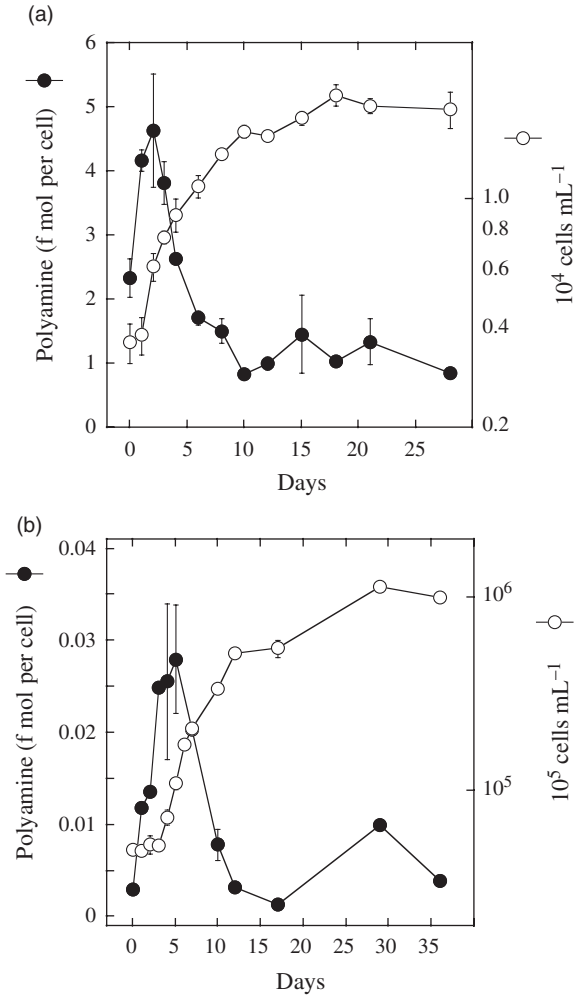

Fig. 5. Changes in (a) caldopentamine contents in *Chattonella antiqua* and (b) homocaldopentamine contents in *Heterosigma akashiwo* during growth.

homocaldopentamine was higher in the exponential phase and was not detected in the lag and the stationary phases.

Discussion

Two raphidophyte species, *C. antiqua* and *H. akashiwo*, contained spermidine as a major polyamine as shown in a previous study (Nishibori & Nishijima, 2004; Nishibori *et al.*, 2006). Although spermine was detected only in *C. antiqua*, trace amounts of putrescine, diaminopropane, and norspermine were common components detected in both species of *Raphidophyceae*. The long linear polyamines, caldopentamine and homocaldopentamine, were among the major polyamine components in *C. antiqua* and *H. akashiwo*, respectively. In the toxic dinoflagellate *Alexandrium minutum*, norspermidine, spermidine and spermine were rather major components and cadaverine and putrescine were the minor polyamines (Lu & Hwang, 2002). Putrescine and spermidine and a trace amount of spermine were the components in the diatom *Skeletonema costatum* (Scoccianti *et al.*, 1995). Thus, the polyamine distribution patterns were different among tested phytoplankton species as mentioned by Hamana & Matsuzaki (1985).

The marked increase in free caldopentamine and homocaldopentamine during the exponential growth phase of *C. antiqua* and *H. akashiwo*, respectively, shown in this study, suggests the relation of these polyamines with growth. Free spermidine also plays a significant role in the growth of these algae (Nishibori & Nishijima, 2004; Nishibori *et al.*, 2006). High polyamine synthetic activity, and as a consequence, high polyamine content in actively growing cells have been reported in many organisms (Tabot & Tabor, 1984). Active polyamine biosynthesis as a response to stress, such as salinity and temperature, has also been reported in many organisms including algae (Scoccianti *et al.*, 1995; Lee, 1998). Although it has thus far been detected only in thermophilic bacteria, homocaldopentamine was detected as an enzymatic reaction product of osmotic-tolerant alfalfa (Bagga *et al.*, 1997). Trace amounts of caldopentamine were detected from the aquatic plants, *Spirodela polyrhiza* and *Pistia stratiotes*, collected from nature (Hamana *et al.*, 1994) and was also detected from drought-tolerant alfalfa and heat-tolerant cotton (Kuehn *et al.*, 1990a). Although the precise function of these polyamines in cells is unknown, these uncommon polyamines seem to have some association with adaptation to adverse environments.

The synthetic pathway of the long linear polyamines have been proposed to be from norspermidine [NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2; 3-3] through norspermine (3-3-3) to caldopentamine (3-3-3-3) (Kuehn *et al.*, 1990b), and from spermidine (3-4) through thermospermine (3-3-4) to homocaldopentamine (3-3-3-4) (Bagga *et al.*, 1997), respectively. Trace amounts of norspermine were detected, but its supposed precursor norspermidine was not detected (detection limit = 0.6 pmol, 0.03 fmol per cell) in *C. antiqua*. In *H. akashiwo*, spermidine was the most abundant polyamine, but the supposed precursor of homocaldopentamine, thermospermine, was not detected (detection limit = 1.0 pmol, 8×10^{-4} fmol per cell).

The detection of caldopentamine and homocaldopentamine in axenic cultures of *C. antiqua* and *H. akashiwo* are the first records of long linear polyamines in unicellular microalgae, implying that the occurrence of these uncommon polyamines is not restricted within the thermophilic organisms and could have a relatively wide distribution in nature.

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